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A MICROCHEMICAL STUDY OF THE FRUIT COAT OF NELUMBO LUTEA

DISSERTATION

Presented in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Graduate School of the
Ohio State University

By

Margaret Fenton Shaw, B.A., M.S.

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PREFACE

This investigation was undertaken to determine the conditions found in the fruit coat of *Nelumbo lutea* which prevented these fruits from germinating. It became necessary to determine the composition of the membrane substances of the fruit coat microchemically in order to interpret the results of the germination tests which were carried on. As the investigation progressed it was extended to include the determination of the nature of the light-zone and also the refractive indices of cellulose present in the palisade layers of the fruit coat.

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A MICROCHEMICAL STUDY OF THE FRUIT COAT OF *NELUMBO LUTEA* [1]

MARGARET FENTON SHAW

(Received for publication December 18, 1928)

INTRODUCTION

This study of *Nelumbo lutea* was begun as a problem in germination, which in these fruits is indefinitely delayed by the presence of the fruit coat. Fruits have been kept in distilled water in this laboratory for two years without showing any swelling or any evidence of germination, while if a cut was made with a file through the fruit coat, germination took place in a few days; proving that some layer of the fruit coat was the retarding factor. Consequently a microchemical analysis of the membrane substances present in the fruit coat was made without considering the integuments or the developing embryo.

HISTORICAL

Most of the literature on species of *Nelumbo* emphasizes the development of the embryo with little or no mention of the character of the fruit coat. Wettstein (20) in 1888 described the anatomy of the fruit coat of the Indian lotus (*Nelumbo nucifera*). The anatomy of the fruit coat of *N. nucifera* differs from that of *N. lutea* only in details, but the two coats according to Wettstein must differ decidedly in their permeability to water. Wettstein reported that the fruit coats of *N. nucifera* swelled greatly in water. He mentioned the entrance of water through the opening at the micropyle, and mentioned "penetration points" of the epidermis as causing a softening and swelling of the fruit. When *N. lutea* is placed in water the fruit coat remains hard and horny, softening only when bacterial action takes place, and then very slowly.

During the same year Wigand and Dennert (21) published a monograph on *Nelumbo speciosum* in which the development of the different layers of the fruit coat was described. Weberbauer (19) in 1893 described the anatomy

[1] Papers from the Department of Botany, The Ohio State University, no. 209.

of the Nymphaeaceae, and included a discussion of the structure of the fruit coat of *N. nucifera*. In 1926, Ohga (13) studied the structure and viability of fruits of *N. nucifera* found buried in a peat bed in Mongolia. The different tissues forming the fruit coats of *N. speciosum* and *N. nucifera*, both old and modern, are very similar to those of *N. lutea*. The work of the previous investigators, mainly descriptive, will be cited only where there are important differences, or where the interpretation of the facts, as observed, disagree.

Collection and Preservation of Material

The material used in this study was collected from Buckeye Lake, Ohio, during the months of July, August, September, and October 1926. A series of collections was made from buds less than an inch long to fruits in dry receptacles. The green material was preserved in glycerin, in water-alcohol-formalin (6 : 3 : 1), and in 70% alcohol, both as sections and as halved fruits. Chromo-acetic acid was tried as a preservative, but did not give good results. It was found necessary to cut the fruits, as the reagents would not enter through the well defined cuticle which was present even on the youngest specimens. There seemed to be no convenient way of telling the age of the fruit for there was such a great variation in the rate of their growth in a single receptacle that dating the flowers would have been of little use. The mature fruits were preserved and allowed to dry in the receptacle.

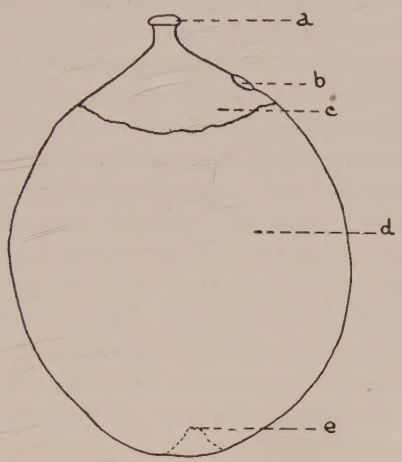
General Description of the Fruit Coat

The fruits of *Nelumbo lutea* when mature are green in color and somewhat rubbery in consistency, turning brown and horny on drying in the air. They are oval in shape, and when dry are about 15 mm. long from the tip of the stigma to the point of attachment, and about 11 mm. wide. The fruit may be divided roughly into two areas: (1) the *cap*, which is more conspicuous in the young stages, bears the stigma and style at its tip and also the protuberance, a bulge near one side of the style; and (2) the *body* of the fruit at the base of which is the point of attachment of the fruit to the receptacle (text fig. 1). Only the tissues of the body of the fruit will be described, as it is less specialized and can therefore be taken as a means of comparison with the more specialized parts of the cap which contain the same tissues with certain modifications. The body of the fruit consists of five distinct tissues which occur in the following order: an outer epidermis, a palisade layer, sclerenchyma, parenchyma, and an inner epidermis.

The epidermis consists of a single layer of cells covered with a thick rubbery cuticle. (Pl. XIX, fig. 1, and Pl. XX, fig. 11.) Ohga described no epidermis at all on the old *Nelumbo* fruits for he believed that it had been worn off. From my own observations of the coats of some of these

old fruits, there seems to be an epidermis, at least over a part of the surface. The stomatal pits can be plainly seen without magnification. Those are the so-called "penetration points" of the epidermis mentioned by Wettstein, which he believed allowed the entrance of water into the fruit, causing germination.

The palisade layer lying directly beneath the epidermis is the most important tissue of the fruit coat physiologically. It is a layer of hyaline cells, generally five- or six-sided in cross section, but very long and slender in longitudinal section (Pl. XIX, fig. 1, and Pl. XX, figs. 14 and 15). (In this report cross sections refer to surface sections of the fruit, while longitudinal sections refer to sections cut along a radius of the fruit.) The



TEXT FIG. 1. Surface view of a mature fruit of *Nelumbo lutea*. *a*, stigma; *b*, protuberance; *c*, cap; *d*, body; *e*, point of attachment.

average length of palisade cells is 74 μ and their width 6 μ. The outer end, *i.e.* the end joining the epidermis, is flattened, while the inner end tapers irregularly. Across the middle of these palisade cells is the light-zone which is a bright band running parallel to the surface of the fruit except where the stomatal cavities cut through, at which points it bends outward (fig. 1). These cells are thick walled with very small lumina. Their compressed cell contents appear to have a disc-shaped end and irregular rod-shaped extension which is continuous through the entire length of the cell without any change in size that would account for the light-zone (fig. 6). This peculiar shape is caused by the thickening of the radial walls leaving a flat layer of protoplasm adjacent to the outer end wall which is unthickened. The width of the palisade cells is uniform except at the light-zone on either side of which is a slight constriction.

The sclerenchyma just beneath the palisade layer is from two to two and a half times the width of the palisade layer, and is composed of thick-walled cells, the outermost layer of which dove-tails with the pointed-tipped palisade cells. These cells are sometimes one-half the length of the palisade cells in size, and have large numbers of canals through the heavy walls connecting the cell cavity to those of the neighboring cells (fig. 3). The sclerenchyma cells are very closely packed together except in places where there are canal-like air spaces winding between the cells and connecting with the stomatal cavities.

The parenchyma lying beneath the sclerenchyma is very similar to parenchyma in general. Its total width is approximately the same as that of the sclerenchyma, but it is composed of thin-walled cells, loosely placed with large air spaces, and contains well developed vascular tissue.

The inner epidermis is a single layer of cells lining the inside of the fruit coat. This layer contains stomata even in the young stages.

The tissues in the region of the cap are heavier than the tissues of the body of the fruit. The epidermis may be one or two cells wide, but in either case it is thicker, with heavier walls. Stomata are also present. The shape of the epidermal cells of the cap differs from that of the body of the fruit, because of a prominent bulge in the outer wall of each cell. This gives a granular appearance to the surface. There is also a white 'bloom" on the surface of the cap which Wigand and Dennert considered due to white crystals or to the stomata. If the bloom is due to stomata, the stomatal pits of the body of the fruit ought to give the same effect since the stomata are similarly placed in that area. The abundant crystals in the epidermis of the cap of *N. lutea* are large aggregates of calcium oxalate, each within a fold of cuticle (fig. 4).

The stigma and the style in the mature fruit form a small, dry, persistent knob, the base of which is cut off from the rest of the fruit by a line of palisade cells which is a continuation of the palisade layer in the cap (Pl. XX, figs. 12, 13 and 14).

The protuberance is a bulge located near the lower edge of the cap (fig. 10) and appears to be a scar with a depression in its center (fig. 11). The epidermis is much thicker across this protuberance, being as much as 4 or 5 cells thick, and containing no stomata or crystals. The palisade layer is narrower directly across the depression, and is made up in thickness of 2 or 3 shorter cells. The light-zone in the palisade layer does not cross this depression, neither do the palisade cells show the bulge in their center which is characteristic of this layer in all other parts of the fruit. There are other differences in the structure of the protuberance, but as they have no special significance they will not be discussed here.

At the end of the fruit opposite the stigma is a conspicuous depression, at the base of which the fruit was attached to the receptacle. There is no definite abscission layer found, as the fruit merely breaks off, often leaving

a ragged plug of vascular tissue protruding (fig. 15). The tissues lining this depression are similar to those found in the body of the fruit.

Microchemical Tests

In order to study the chemical differences in the tissues of the fruit coat it was necessary to employ microchemical tests. An effort was made to employ a sufficient number of different critical tests for each membrane substance to determine its presence or absence with certainty when occurring in sufficient amounts to be detected by the methods used. Furthermore, the composition of the mature membranes, particularly those that have been lignified, cutinized, or suberized, cannot always be readily determined without a knowledge of their development from a younger state. Consequently tests were used upon the various tissues of the fruit coats during several stages of development, the first tests being made on young tissues when the cell walls were composed mainly of pectic compounds and cellulose.

The microchemical tests used in this study may be classified into several groups. One group of tests involved color reaction; the simplest type is a process of staining by which the dye is differentially absorbed by the various membrane substances. A second group of color reactions are those in which physical or chemical alterations of the membrane substances are involved without destroying the membrane, at least until observations can be made. Examples of this type are the hydrocellulose reaction for cellulose, the chitosan test for chitin, and the phloroglucin-hydrochloric acid test for lignin. These color reactions were used only as indicators to show, in general, the position of certain substances. In some cases they made layers stand out by contrast, but final conclusions were not based upon them. In cases where lamellae are very thin, there are many complications due to light refraction causing colored light fringes around the object, which confuse the results of the color reactions.

Solubility tests are among the most reliable ones. They may be used in two ways when dealing with membrane substances. First, a substance such as cellulose may be dissolved out of a cell, and its absence checked by other tests; or second, all other substances around the cellulose may be removed, leaving only the cellulose, which may be then checked by other tests.

Still another group of tests are those in which some chemical change takes place which does not necessarily remove the substance, but may only change its character. Among these reactions are the ceric and phellonic acid reactions for suberin. Still more drastic chemical alterations may be made in tests for certain membrane substances, such as the complete hydrolysis followed by tests for the ultimate degradation products.

Since the refractive index of a substance is always the same under the same experimental conditions, it can be used as a means of identifying the substances in cell membranes. Those indices are obtained by the Becke

method (Kraus and Hill, 8) by means of a series of oils standardized by a refractometer, applied to perfectly dry material. This is necessary because the water content of a substance would vary the refractive index. The number of indices of a substance found along different axes is a very important part of the identification of a substance, since this indicates to which system of crystals the material belongs. In this report the index of refraction was used as a means of identifying the cellulose in certain tissues.

One of the most reliable checks for the position of different substances is that furnished by the use of the polarizing microscope. All crystalline substances and substances under strain are doubly refracting (bright between crossed nicols), while amorphous substances not under strain are singly refracting (dark between crossed nicols). For this study a petrographic microscope was used with a Bausch and Lomb projecting lantern for light. Ordinary lighting was not sufficient to bring out the thin lamellae clearly. In cases where the double refraction is so weak as to be doubtful, the selenite plate was found to be very helpful. The addition of the first order red of this plate raises the color of part of the doubly refracting substances to blue in the scale of interference colors, and lowers the rest to yellow, but it leaves any amorphous substance red (Chamot, 3).

In this investigation the standard microchemical tests given by Molisch (11), Tunmann (17), and Eckerson (5) were used. Tests not given by these authors are cited separately.

The term "lignification" is used for cell membranes which give a positive reaction to the Maule test for lignin and the phloroglucin-hydrochloric acid test, and which also show positive results with lignin solvents.

Tests Used for Cellulose

(1) Color reactions: congo red, methylene blue, chlorozinc iodid; (2) Soluble in copper-oxid-ammonia, 75% sulfuric acid, 50% chromic acid, and also in dilute chromic acid, if allowed to act for a week or more; (3) Insoluble in Schultze's reagent, boiling glycerin, concentrated potassium hydroxid; (4) Hydrocellulose reaction; (5) Refractive indices; (6) Polarized light; bright between crossed nicols (figs. 4 and 7).

Tests Used for Hemicelluloses

These were all negative. (1) Soluble in boiling 3% sulfuric acid, boiling glycerin at 210° (Schorger, 15); (2) Polarized light: in all preliminary tests made on very thin sections the hemicelluloses were dark between crossed nicols.

Tests for Pectic Compounds

General tests: (1) Color reactions: ruthenium red, methylene blue; (2) Polarized light: pectic compounds are amorphous substances, always being dark between crossed nicols under the polarizer; (3) Solubility: distinguished

from cellulose by insolubility in copper-oxid-ammonia and from cellulose, suberin, cutin and lignin by its solubility in hot dilute acids followed by dilute alkali.

The criteria used for distinguishing different forms of pectic compounds are as follows: *Pectin*, soluble in water. *Pectic acid*, insoluble in water, soluble in dilute alkalies, ammonia and alkali carbonates. *Pectose*, (1) hydrolized by 2% hydrochloric acid to pectic acid or pectin; (2) insoluble in water, dilute alkalies, ammonia, alkali carbonates. *Calcium pectate*, (1) hydrolized by 2% hydrochloric acid; calcium chlorid is formed and pectic acid set free; (2) 3% oxalic acid or ammonium oxalate; calcium oxalate crystals are formed and pectic acid set free; (3) 5% sulfuric acid: calcium sulfate crystals are formed and pectic acid set free; (4) insoluble in water, dilute alkalies, ammonia, alkali carbonates.

Tests Used for Callose

These were all negative. (1) Color reactions: resorcin blue, aniline blue, rosolic acid; (2) Polarized light: dark between crossed nicols.

Tests Used for Lignified Membranes

(1) Color reactions: phloroglucin-hydrochloric acid, Maule's reaction, potassium permanganate-hydrochloric acid-ammonia; (Maule, 10); (2) Soluble in 50% chromic acid, Schultze's reagent, hydrogen peroxid; (3) Insoluble in copper-oxid-ammonia, 75% sulfuric acid, potassium hydroxid, sodium hydroxid; (4) Polarized light: the lignin-pectic lamellae were dark between crossed nicols; the lignin-cellulose lamellae were bright between crossed nicols.

Tests Used for Chitin

(1) Color reactions: chitosan reactions, dilute sulfuric acid and sodium chlorid (van Wisselingh, 22); picric acid (van Wisselingh).

Tests for Cutin and Suberin

(1) Color reactions: Scharlach red, Sudan III, osmic acid, chlorophyll solutions in alcohol and acetone; these chlorophyll solutions gave very uncertain results, and not sufficient contrast for use with very thin lamellae; (2) Soluble in 3% alcoholic potassium hydroxid, 2–5% potassium or sodium hydroxid, 10% potassium hydroxid in glycerin, warmed (van Wisselingh); (3) Insoluble in copper-oxid-ammonia, 75% sulfuric acid, 50% chromic acid, Schultze's reagent in the cold; (4) Ceric acid reaction; Poulsen's (14) modification of this reaction was used as follows: the tissue was treated with Schultze's reagent for a few minutes in the cold, then warmed slightly with potash; Poulsen advocated this modification for use with membranes "only slightly suberiferous," as they would otherwise scarcely show the reaction; (5) Polarized light: bright between crossed nicols; the cuticle and the suberin lamellae appear bright yellow between crossed nicols, in contrast to the brilliant white of the cellulose.

The two following tests were used to distinguish suberin from cutin: (1) Boiling glycerin 256°–300° C. This high temperature was obtained by heating the sections in a sealed tube in an oil bath (van Wisselingh). Paraffin was used for the oil bath. (2) Phellonic acid reaction. To obtain any positive result in this test, it was necessary to remove the cellulose first, as the chlorozinc iodid turned the cellulose such a deep color that it masked the violet color due to the potassium phellonate. It was found that 75% sulfuric acid was best to remove the cellulose. Chromic acid was not so useful because it left only the suberin lamellae which wash away, and copper-oxid-ammonia gave negative results. According to Gilson (6) the copper unites with the phellonic acid to form a compound not colored violet with iodine.

Composition of the Cell Walls

All the mature tissues of *Nelumbo lutea* contain a middle lamella of a mixture of pectic compounds and lignin. This lamella is dark under the polarizing microscope, indicating the presence and predominance of amorphous substances. Lignification of pectic lamellae obscures the usual tests for pectic compounds just as it obscures the usual tests for cellulose in lignified cellulose lamellae. Each cell also has a cellulose lamella which is present on each side of the middle lamella and remains throughout the growth of the cell. The epidermis, palisade layer, and sclerenchyma have additional thickenings of cellulose, which in the palisade cells and sclerenchyma are very heavy.

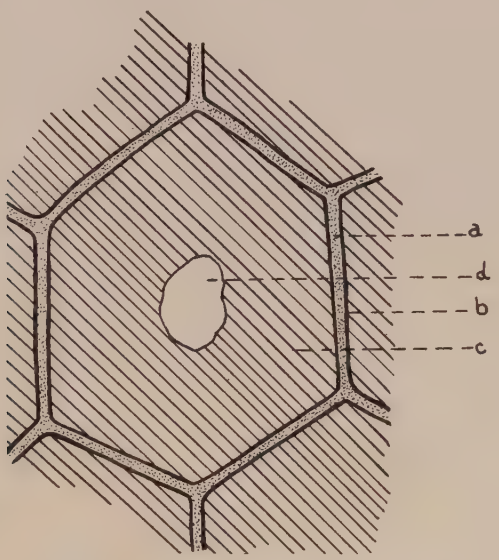
Variations of the arrangement of these lamellae are found in the epidermis, which has a thicker layer of cuticle deposited in a continuous sheet over the outer surface of the cells; and in the palisade layer, which has a deposit of suberin in a lamella between the cellulose lamella and the lignified middle lamella (text fig. 2 and Pl. XX, figs. 8 and 9). This suberin lamella is continuous over the entire surface of the cell, except at irregularly placed pits in the wall caused by intercellular connections, and at the pore canals in the region of the light-zone.

Most of the difficulty in this study was due to the presence of adjacent lamellae of lignin and suberin in the palisade cells. The lignified middle lamella prevented the cells from being crushed or teased apart: the suberin lamella formed a water-proof covering over the inner cellulose layers resisting completely the penetration of nearly all cellulose reagents. This difficulty was met in two ways: first, by cutting surface sections of the fruit coat, which made cross sections of each cell and exposed the different lamellae to the reagents; and second, by isolating the palisade cells by dissolving out the lignified middle lamella, which then allowed the reagents to act directly on the suberin and indirectly on the cellulose through the intercellular connections.

The middle lamella was stained best by the Maule reaction, and only

slightly by the phloroglucin-hydrochloric acid test. It was removed by all the lignin solvents mentioned, thereby separating the palisade cells, since the lignin solvents used also dissolved any pectic compounds that were present.

The suberized lamella, because of its thinness, was very difficult to see. The usual stains, as Scharlach red and Sudan III, showed only the general position of this lamella by the color present, but 1% osmic acid showed the suberized lamellae as two distinct lines on either side of the middle lamella. The polarizing microscope also showed these lamellae as bright lines on either side of the dark line of the middle lamella, and outside of the heavy cellulose. The irridescent colors which appear both in parallel and



TEXT FIG. 2. Diagram of a cross section of a mature palisade cell of *Nelumbo lutea*. *a*, middle lamella, a pectic-lignin complex; *b*, secondary suberin lamella; *c*, secondary cellulose lamella; *d*, lumen of cell.

convergent polarized light are due to the thin fatty layer of suberin around the cellulose. The presence of the suberin lamella was most accurately demonstrated by dissolving all other substances away by 50% chromic acid. After this treatment only the cuticle and suberin were left, the latter being then only a thin sheath which could be stained with Sudan III or osmic acid. Throughout the study chromic acid was found to be the most reliable check of the efficiency of any solvent for cutin or suberin. When the solvent had been effective, and the suberin or cutin broken down, the chromic acid would dissolve away the entire tissue in a few minutes. However, if the suberin or cutin were not broken down, they remained as distinct thin lines after the rest of the cells had disappeared. In the hydrocellulose

reaction on isolated uncut cells, the suberin lamella retarded the reaction. The hydrocellulose reagents finally entered perhaps through the intercellular connections. Once the hydrocellulose action was started the swelling of the cellulose caused this impervious sheath of suberin to give way in places, but where unbroken it formed constrictions, binding the swelling cellulose for a time, and producing a peculiar beaded effect. When the cellulose was at last completely swollen, the suberin lamella would still be visible as a torn shread of clear yellow material.

These two reactions demonstrated the existence of the suberin, but not its position in the wall in relation to the rest of the cell. Cross sections treated with 75% sulfuric acid removed the cellulose leaving a mesh-like structure, consisting of the middle lamella plus the suberin lamellae on either side of it (fig. 9). The middle lamella could then be dissolved out slowly with hydrogen peroxid which left the suberin lamellae as thin rings, remaining in their original positions. The same result was obtained after treatment with copper-oxid-ammonia to remove the cellulose, and 50% chromic acid to remove the middle lamella.

When the palisade cells were observed under the polarizer in longitudinal section they were extremely bright, indicating a high birefringence (fig. 7), while very thin cross sections of the cellulose layer appear nearly black, indicating a low birefringence. In thicker sections, and sections not quite perpendicular to the long axis of the cell, the cellulose was brighter, but not as brilliant as that in longitudinal view.

These observations of the birefringence of cellulose in longitudinal and cross sections were explained when the refractive indices were taken. The two indices for the longitudinal section were found to be 1.568 for the ray vibrating parallel to the long axis of the cell, and 1.534 for the ray vibrating at right angles to it. The indices for the two rays vibrating at right angles to each other in the cross section of the cell were found to be 1.535 and 1.531. If the two indices 1.534 and 1.535 are considered as being the same ray, the three indices for cellulose then are 1.568, 1.534, and 1.531. Anderson (2) gave two indices for the cellulose of the bast fibers of flax, namely 1.58 and 1.52. These indices were taken after the material was hydrolized slightly, washed, and dried on a hot plate. The refractive index given by de Mosenthal (12) for the cellulose of wood, ramie, and cotton was 1.5310. In the cellulose of *Nelumbo lutea* the two indices of the longitudinal section of the palisade cells show a high birefringence of 0.034, accounting for the brilliance of the cells between crossed nicols; the birefringence of the cellulose in the cross section is only 0.003, accounting for the darker condition observed there.

Both Herzog (7) and Sponsler (16) in their work with X-ray diffraction patterns of cellulose found that it belonged to the orthorhombic system of crystals. The crystals in this system are biaxial with three axes of different lengths, and with the three different indices. Anderson's two

indices were evidently for the longitudinal view of the bast fibers. One is higher and the other lower than any found in this study. The single index of de Mosenthal agrees with one of the three found for *Nelumbo*.

The Developing Tissues

The tissues of the youngest stage studied, that of an unopened bud about one inch long, show a condition in which all the cells were in a meristematic condition. They have no special arrangement except the two outer layers which are very regular in size and shape, being square and arranged in two rows. These are the cells which will later become the epidermal and palisade cells. The middle lamella of all the cells is pectose, and the thin lamella on either side of it consists of a combination of pectose and cellulose. This pectose-cellulose lamella is dimly bright between crossed nicols with the polarizing microscope, showing that it is a mixture of crystalline and amorphous substances, but the middle lamella is dark.

Further development from this early meristematic condition differs in rate in the different areas of the fruit coat.

The period of greatest cell division took place before the opening of the bud. According to Wigand and Dennert the tissues of the protuberance are first to reach maturity. This area is the first to develop either by enlargement or by differentiation chemically from the meristematic tissue. The maturing of the tissues of the protuberance is followed by that of the stigma and style, the cap, the point of attachment, and, last, the tissues of the body of the fruit.

Differentiation of the Cells

The middle lamella of all meristematic cells of the fruit coat is of pectose. As differentiation proceeds, the middle lamellae of the palisade cells give negative reactions to solvents for pectic substances. The reason for this is because the middle lamella has become lignified. The change from pectose in the middle lamella to a lignin complex is made evident by the failure of the middle lamella to disappear in tests with pectose solvents, and by the positive tests for lignin. This lignification follows the same sequence in the different areas as the growth of the cells, affecting first the protuberance, next the style, then the cap and point of attachment, and finally the tissue of the body.

This lignification is followed soon after by the occurrence of a suberin lamella which appears in each palisade cell between the cellulose lamella and the lignified middle lamella. Whether some lamella already present becomes suberized, or a thin sheet of suberin is deposited between the middle lamella and the cellulose lamella, was not fully determined. Lee (9) in his work on abscission showed that single cells in a mature tissue could become suberized. The suberin lamella in the palisade cell is not complete at first, but begins at both ends and works toward the center.

The area of the cell, where later the light-zone appears, is the last to become suberized.

In addition to the suberin test another verification of the fact that the center of the palisade cells is the last to be suberized is shown by the hydrocellulose reaction on immature palisade cells. Here the center of the palisade cells was attacked first by the reagents, showing that this region of the cell, later to become the light-zone, was less resistant to the penetration of reagents than the rest of the cell. The pressure due to the growth of the surrounding tissue causes this less developed part of the cell to become crushed, leaving it decidedly loose and misshapen. Even when the suberin lamella is complete, this irregularity still remains. This suberin lamella ultimately covers the whole of the palisade cells, except at points irregularly placed where intercellular connections pass through. The presence of the suberin is a criterion of the completion of the growth of the palisade cells in size. However, this suberin lamella is present before the completion of the growth in thickness of the cell wall, as it appears before the heavy layer of cellulose, which practically fills the cell lumen, is laid down.

Suberin formation in the palisade cells occurs first in the protuberance, and then occurs in the palisade cells of the other regions in the same order as that taken by the spread of lignification. Its appearance in the style is worthy of special mention. Just after fertilization a line of cells across the base of the style becomes suberized (fig. 12). The spread of suberization from this point is up into the style, and in the walls of the cells of the epidermis just below the style (fig. 13). The contents of these suberized cells become dark brown. The most effective microchemical test for showing the suberin is to treat the tissue with 50% chromic acid for a few minutes. This breaks down all the cell-membrane substances except the cuticle and the suberin lamella.

The great development of cellulose in the palisade cells takes place immediately after the deposit of the suberin lamella. This development follows the same sequence in its spread through the different regions of the fruit coat as did the lignification and suberization. In the walls of the palisade cells the thickening is on the radial walls only, forming a cylinder with the inner end closed, as the cellulose comes together at that point (fig. 6). The cellulose lamella fills out the irregularity which occurred during the formation of the suberin lamellae. This thick cellulose lamella ultimately occupies nearly the entire space of the lumen, and the cell contents become hard and dry, there being finally only a remnant left. The pore canals pass through the cellulose wall just above and below the light-zone, but the intercellular connections seem to be cut off by the cellulose, as there is no evidence of them in the cross sections. The development of the cellulose in the sclerenchyma is extremely irregular as it follows the contours of the cells including the very distinct intercellular connections which remain (fig. 3).

Attempts made by previous investigators to interpret the chemical nature of the fruit coat of *Nelumbo* appear very unsatisfactory. The indefiniteness and lack of agreement in the literature appear to be the result mainly of attempts to base conclusions upon an insufficient number of critical tests and of failure to recognize the fact that the cell walls of plants usually consist of two or more lamellae differing in chemical constitution.

The Light-zone

A number of workers have been interested in species of *Nelumbo* because of the very striking "light-zone" or "light-line" present in the palisade layer of the fruit coat. Several different families of plants beside the Nymphaeaceae produce seeds with light-zones, the most notable being the Leguminosae, Malvaceae, and Cannaceae. As early as 1846 Mettenius described this peculiarity, and numerous investigators have attempted to determine the nature of the light-zone.

Coe and Martin (4) presented in their historical discussion a brief summary of these different theories of the nature of light-zones. Because these earlier workers each described the light-zone of a different seed, and as no one has examined all types, the data which can be collected are very meager and scattered. Very few of these investigators traced the development of the light-zone through the young stages of the seed, a method which might have thrown light on the origin of certain of the peculiarities found in this band. In this study only work with species of *Nelumbo* will be cited.

Even among the four investigators previously mentioned, who worked with *Nelumbo*, there is a great variety of opinions as to the cause of the light-zone. Both Wettstein and Ohga mentioned peculiar staining effects, without a clear appreciation of the effect of lignified and suberized lamellae on the entrance of reagents. Wettstein noted the fact that often treatment with concentrated potassium hydroxid, either boiling or cold, caused the light-zone to disappear. It was found that this treatment destroys the suberin lamella, causing the cells to separate; and, as will be explained later, isolated cells do not show the light-zone in all cases. He believed that the reason why this zone behaved so peculiarly to reagents was because the cell wall was thickest and most impermeable at the light-zone. He explained the light-zone as due to chemical and physical differences at this point. Wigand and Dennert stated definitely that the brightness of the light-zone was not because of lignification, and that it was not water-poor, but did not present evidence for the latter fact. The refractive indices of this bright line are no different than for the rest of the palisade cell, which would corroborate the statement that it is not relatively water-poor, since the refractive indices would change with the water content.

These workers believed that the region of the light-zone was warped and distorted because of the presence of the pore canals which made the

area less firm. There is difference of opinion as to the character of these pore canals. Weberbauer and Wigand and Dennert believed that they crossed the light-zone, while Wettstein and Ohga regarded the light-zone as clear, with the pore canals forming a darker band on either side. They stated that the cause of the light-zone was due to these pore canals. Ohga, without presenting data, states (p. 12) that the light-zone is due to the cell contents. On the same page he says that "the nature or structure of this zone could not be determined." Wettstein, however, maintained that the light-zone appeared brighter by contrast with the darker line of the pore canals.

In the young stages the protoplasm gathers together in the center of the cells, at about the place where the light-zone later appears. This fact was noted by Wigand and Dennert who believed that it always appeared different from the rest of the cell. These aggregates, although they are at nearly the same level in the cells, still do not hold as definite and clear-cut a line as that of the light-zone. They are truly cell contents, as they can be cleared out of the cells with potassium hydroxid or chloral hydrate.

In the observations made on the growth of the tissues earlier in this study, the light-zone is first evident at the time of development of the suberin lamella, which is deposited first at the ends of the palisade cells, and leaves the central portion unsuberized. This region crushes and becomes loose and distorted in appearance. The deposit of the cellulose appears to fill up the spaces thus formed, causing a slight though perceptible enlargement at that point (Pl. XIX, figs. 2 and 7). The pore canals develop on either side of this enlargement, emphasizing it.

In the mature fruit the lignified, suberized, and cellulose lamellae are similar throughout the entire length of the cell, showing no difference at all in the chemical nature of the light-zone (fig. 6). When single palisade cells are isolated, the brightness of the light-zone disappears, but it can be made to reappear if the single cell is put a trifle out of focus. In fact, by changing the focus slightly light-zones can be made to appear on any curved surface of the cell. If the cell is turned in a certain position, there will be a light-zone on the curve, along the entire length of the cell (fig. 2). According to the position of the cell, light-zones can be produced on the ends of the cell, or on the curved sides of the pore canals opposite the central enlargement.

It is concluded from these observations that in *Nelumbo lutea* the region of the light-zone, being perceptibly larger than the constrictions at the pore canals, acts as a convex lens, bringing more light rays to a focus at this point than in regions immediately adjacent to it. This would cause a brighter area in the center of an otherwise uniformly straight cell. No chemical difference from the rest of the cell was found at this point.

Experiments in Germinating Fruits

Wettstein's observation, mentioned earlier in the paper, indicated that *Nelumbo nucifera* was germinated easily. He tried experiments with sections placed in dyes and found that the dyes were absorbed on both sides of the light-zone first. From this he concluded that the water entered through the stomatal canals, and passed around the fruit coat on either side of the light-zone forming a "water sheath" surrounding the embryo with moisture. The absorption of dye along the light-zone by a section of the fruit coat is quite common, as there are intercellular spaces caused by the constriction at the pore canals, which act as pockets collecting air and dyes (fig. 1). These are quite conspicuous. In the whole fruit, however, the water does not pass into the interior through the stomatal cavities. This will be discussed more in detail later.

Wettstein, working with *N. nucifera*, describes the papillae which line the depression at the top of the stigma as "a brown felt-like tissue." He also claimed that this tissue lined the stylar canal and that its cells "quickly fill up with water." He thought that the water also entered the fruit by this means. In *N. lutea* these papillae cover the surface of the stigma and stylar canal, and the attachment of the ovule (fig. 10), and are surrounded by a brown jelly-like substance. However, no evidence of their swelling unusually has been noted. The style is cut off from the rest of the fruit by layers of suberized cells as has already been mentioned.

Ohga's work on the ancient fruits of *N. nucifera* showed that some layer of tissue prevented the entrance of water in these also. Ohga experimented with the removal of the coat with strong acids and alkalies. He found concentrated sulfuric acid to be the most satisfactory, and states that "as long as the acid does not reach to the hyaline zone (light-zone) the water is hardly absorbed at all by the fruit, but once this hyaline zone is removed by it (the acid) the water can go in freely." From this it appears that he believes the light-zone to be the impermeable layer.

The results of the microchemical study of the fruit coat indicate that the palisade layer as a whole and not the light-zone prevents the entrance of water. To test this hypothesis fruits were filed at different levels through the fruit coat. Some of these cuts extended to the light-zone, others just through it, and still others through the palisade layer. After the fruits had had time to germinate, the coats were examined in sections taken across the cut, and it was found that only those germinated in which the cut went completely through the palisade layer. This shows that the palisade layer is the impermeable layer, and not the light-zone.

The reason for water not entering the large stomatal openings, 4–22 μ in width, which penetrate this impervious layer, was next considered (figs. 1 and 5). As there was always a large amount of air found in the tissue, it was thought that these openings might be plugged up with air. To remedy this, fruits were placed in distilled water and the air extracted

with a pressure pump until no more bubbles appeared. The fruits were left in the water to germinate, but no germination resulted.

Ursprung's (18) work on the penetration of liquid into intercellular spaces seemed to give the best explanation of these results. He states that thin films of fatty substances lining air spaces will prevent the entrance of water. The only possible way to cause water to enter these stomatal cavities in *N. lutea* is to remove the fatty suberin lamella by some solvent. Aside from this method the only way to germinate these fruits is by filing the coats through the palisade layer, or by removing this layer by strong reagents.

As a preliminary test on the effect of fat solvents a few fruits were placed in alcohol, ether, chloroform, xylol, and acetone for an hour, washed, and placed in distilled water. After a few days those placed in acetone swelled, but did not germinate because the embryos were killed, while one or two germinated which were treated by ether. None of the others showed any signs of softening. Perhaps a more extensive study of the effects of fat solvents is worthy of consideration.

Summary

1. The palisade layer of *Nelumbo lutea*, and not the light-zone, is the impermeable layer of the fruit coat which prevents the entrance of water into the fruit.

2. The resistance of the palisade layer to reagents is due to a secondary lamella of suberin which surrounds the heavy secondary cellulose wall.

3. The stomatal openings do not admit water into the inner tissues because of the suberin lamella surrounding each palisade cell lining the stomatal cavity.

4. Treating the fruits with some fat solvent, as ether, will allow the entrance of water, followed by germination.

5. The light-zone in the palisade cells is due to a bulge in the cells at that point, which acts as a convex lens, bringing more light rays to a focus there.

6. In the palisade cells in the depression of the protuberance there is no bulge and no light-zone.

7. The palisade cells when isolated show no brighter, more doubly refractive band at the point of the light-zone than elsewhere, but only a swelling at that point with pore canals on either side.

8. Bright bands similar to the light-zone can be seen on any curved surface of the cell in certain positions, such as the ends or along the whole length of the cell.

9. The differentiation of the palisade cells, after their growth in size is complete, occurs in the following order: lignification of the middle lamella; suberization or the formation of a suberin lamella adjacent to the middle lamella; formation of a thick lamella of cellulose which fills most of the cell lumen.

10. The above changes appear first in the protuberance, next in the base of the style, then the cap and point of attachment simultaneously, and last in the body of the fruit.

11. The refractive indices of the heavy cellulose lamella in the palisade layer were found to be 1.538, 1.534, and 1.531.

12. Longitudinal sections of the palisade cells between crossed nicols under the polarizer show very bright, due to the high birefringence of the two rays in this plane vibrating at right angles.

13. Cross sections of the palisade cells, however, show a nearly dark field because of the low birefringence of the rays in this plane vibrating at right angles to each other.

The writer wishes to acknowledge her indebtedness to Dr. H. C. Sampson and Dr. S. D. Sayre of the Department of Botany of the Ohio State University for many helpful suggestions and criticisms of the microchemical study; and to express her appreciation for the help and criticisms of Dr. W. J. McCaughey of the Department of Mineralogy of the same University on the study of the optical properties of these tissues.

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EXPLANATION OF PLATES

Plate XIX

Fig. 1. Longitudinal section through mature fruit coat, showing relation of tissues; epidermis, palisade layer, sclerenchyma, stomatal cavity, light-zone. Note outward bending of light-zone at stoma, also air pockets along light-zone. × 260.

Fig. 2. Palisade cells, showing the possibility of light-zones on any curved surface. In the group and the lower cell, the light-zone is on the end of the cell; in the upper cell the light-zone is along the whole length of the cell. × 260.

Fig. 3. Detail of mature sclerenchyma cells. Note heavy cellulose walls, and dark cell contents with conspicuous intercellular connections. × 260.

Fig. 4. Surface view of epidermis of cap region under polarizing microscope. Cellulose walls and calcium oxalate crystals show bright. × 210, approximately.

Fig. 5. Surface view of epidermis of body, showing stoma with conspicuous guard cells. The stoma is sunken causing the surface of the epidermis to be slightly out of focus. × 260.

Fig. 6. An isolated mature palisade cell with several sclerenchyma cells, showing no evidence of a light-zone, but heavy cellulose on lateral walls, and thin cellulose only on end wall. × 260.

Fig. 7. Isolated mature palisade cells under the polarizing microscope, showing cellulose bright. Note slight bulge in region of the light-zone, but no greater brightness. × 210, approximately.

Plate XX

Fig. 8. Cross section of mature palisade cells, showing middle lamella and suberin lamella as a single dark line, cellulose nearly filling lumen of cell. Stomatal cavity prominent at left. × 260.

Fig. 9. Same as figure 8 after treatment with 75% sulfuric acid; middle lamella and suberin remaining, cellulose dissolved. The breaks in the walls are intercellular connections. × 260.

Fig. 10. Median longitudinal section of young fruit showing protuberance, stigmatic surface, and stylar canal. × 15.

Fig. 11. Longitudinal section of protuberance at the opening of the flower, showing thickening of epidermis and palisade cells. × 15.

Fig. 12. Median longitudinal section of the stylar canal at the opening of the flower. Note dark band of cells across base of style where suberization will later occur. × 15.

Fig. 13. Detail of figure 12, showing junction of epidermis and palisade layer with style. Note no evidence of palisade layer across base of style. × 60.

Fig. 14. Median longitudinal section through stigma and style of mature dry fruit, showing relation of different tissues, development of a palisade layer across the base of the style, stylar canal, and dry suberized portion of style outside the palisade layer. × 15.

Fig. 15. Median longitudinal section through the point of attachment of mature dry fruit, showing relation of different tissues, and great development of vascular tissue. × 15.

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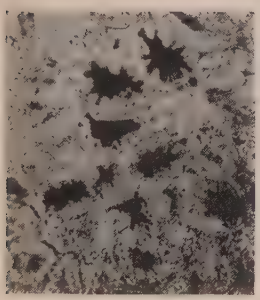

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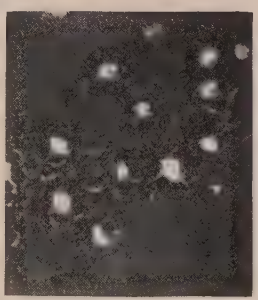

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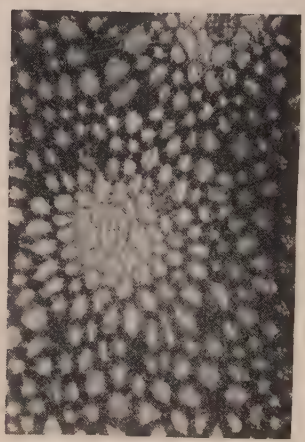

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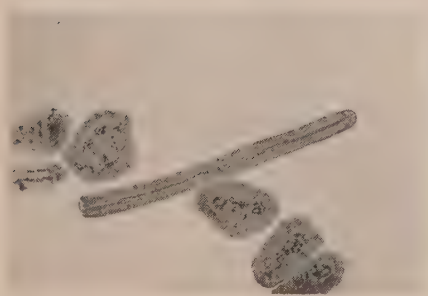

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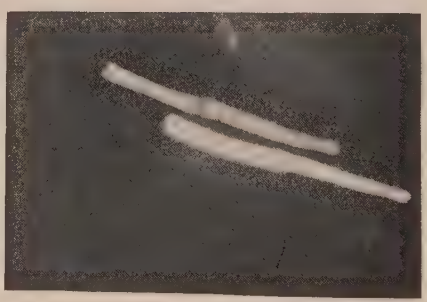

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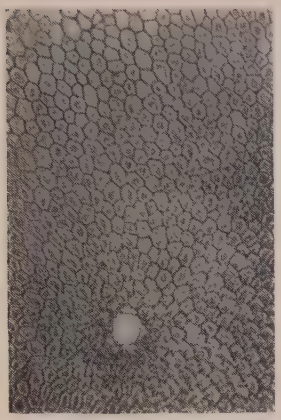

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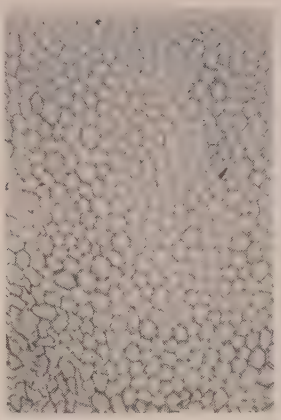

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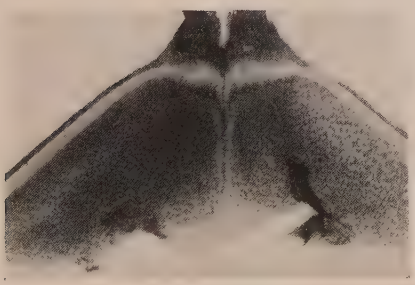

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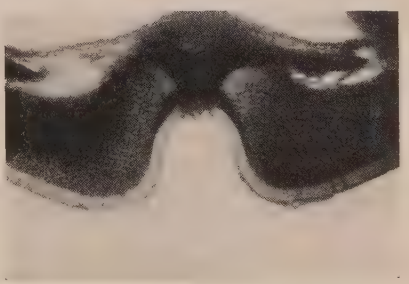

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AUTOBIOGRAPHY

I, Margaret Fenton Shaw, was born in Honolulu, Territory of Hawaii, September 13, 1891. I received all of my secondary education in the schools of Honolulu, with the exception of one year of high school at Forest Grove, Ore. From 1911 to 1915, I was a student at Mount Holyoke College, receiving the degree of Bachelor of Arts in 1915. The following five years were spent in Honolulu, as teacher in the Territorial Normal and Training School, in charge of the teaching of hygiene and sanitation. In 1920, I accepted a position as instructor of botany at Vassar College for one semester, completing the year with study toward the degree of Master of Science at the University of Chicago. In 1921, I returned to Vassar, where I continued my work as instructor in botany until 1924. I completed the work for my degree of Master of Science at the University of Chicago in the summer of 1922, receiving my degree that same year. The summer of 1923 was spent at the Marine Biological Laboratory at Woods Hole, Mass., studying marine flora. From September, 1923, to June, 1925, I held a Graduate Scholarship in Botany at the Ohio State University, devoting full time to graduate study and research leading to the degree of Doctor of Philosophy. From March, 1926, to September, 1927, this work was continued.